AD_____
(Leave blank)

Award Number:
W81XWH-07-1-0645

TITLE:
Nuclear Drug Delivery for Breast Cancer Chemotherapy

PRINCIPAL INVESTIGATOR:
Youqing Shen, Ph.D.

CONTRACTING ORGANIZATION:
University of Wyoming
Laramie, WY 82071

REPORT DATE:
September 2008

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

☒ Approved for public release; distribution unlimited

☐ Distribution limited to U.S. Government agencies only; report contains proprietary information

REPORT DOCUMENTATION PAGE

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
30-09-2008	Annual Report	1 SEP 2007 - 31 AUG 2008

4. TITLE AND SUBTITLE
Nuclear Drug Delivery for Breast Cancer Chemotherapy

5a. CONTRACT NUMBER
W81XWH-07-1-0645

5b. GRANT NUMBER
BC062422

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Shen, Youqing (PI)
Murdoch , William J. (Co-PI)

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Wyoming
1000 E University Ave., Laramie, WY 82071

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Department of Defense (DOD) Breast Cancer Research Program (BCRP) of the Office of the Congressionally Directed Medical Research Programs (CDMRP),

10. SPONSOR/MONITOR'S ACRONYM(S)
CDMRP

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Chemo-resistant breast cancer cells overexpress not only membrane-associated but also many cytosolic drug-resistance mechanisms to limit the access of DNA-toxins to the nucleus, causing low chemotherapeutic efficacy. The project aims to synthesize and evaluate a conjugate that can enter and deliver DNA-toxins doxorubicin (DOX) or camptothecin (CPT) to the nuclei of breast cancer cells. In this project, a polyethyleneimine (PEI) was used as a nuclear localizing carrier, and was functionalized with folic acid as targeting groups and CPT as a DNA-toxin. The PEI carrier was modified to be negatively charged in the bloodstream to shield its cationic charges and thereby inhibit its interaction with cells before reaching tumors, but once in acidic tumor interstitium or tumor cells' acidic lysosomes, the PEI is regenerate for cellular uptake and nuclear localization. The PEI-based conjugate could enter the breast cancer cells efficiently and traverse to their nuclei. The carried CPT showed much higher cytotoxicity to breast cancer cells than CPT itself. Similarly, a cationic polylysine-based conjugate was also synthesized and similar results were obtained.

15. SUBJECT TERMS
Polymer-drug conjugate, nuclear drug delivery, drug resistance, breast cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER

Table of Contents

Nuclear Drug Delivery for Breast Cancer Chemotherapy

Youqing Shen, Ph.D. (PI), William J. Murdoch, Ph.D. (Co-PI)

Introduction:

The project was carried out in the past one-year according to the Statement of Work shown as follows.

TASK 1. To synthesize and characterize folic-acid– or LHRH-functionalized charge reversal conjugates (5 Months):

a. Synthesize linear polyethyleneimine (PEI, Mn ~5-10kDa) by ring-opening polymerization.
b. React the PEI with proper 5-membered ring-anhydrides to prepare charge-reversal PEIs (CR-PEIs), characterize and optimize their charge-reversal kinetics.
c. Introduce folic acid or LHRH to the CR-PEI using a post-reaction method.
d. Introduce doxorubicin (DOX) or camptothecin (CPT) via a disulfide bond to the targeting group-functionalized CR-PEI.

Milestone 1: Obtaining the FA- or LHRH-functionalized TCRC with optimal charge-reversal kinetics.

TASK 2. To in vitro and in vivo evaluate the TCRCs for breast cancer chemotherapy (7 Months):

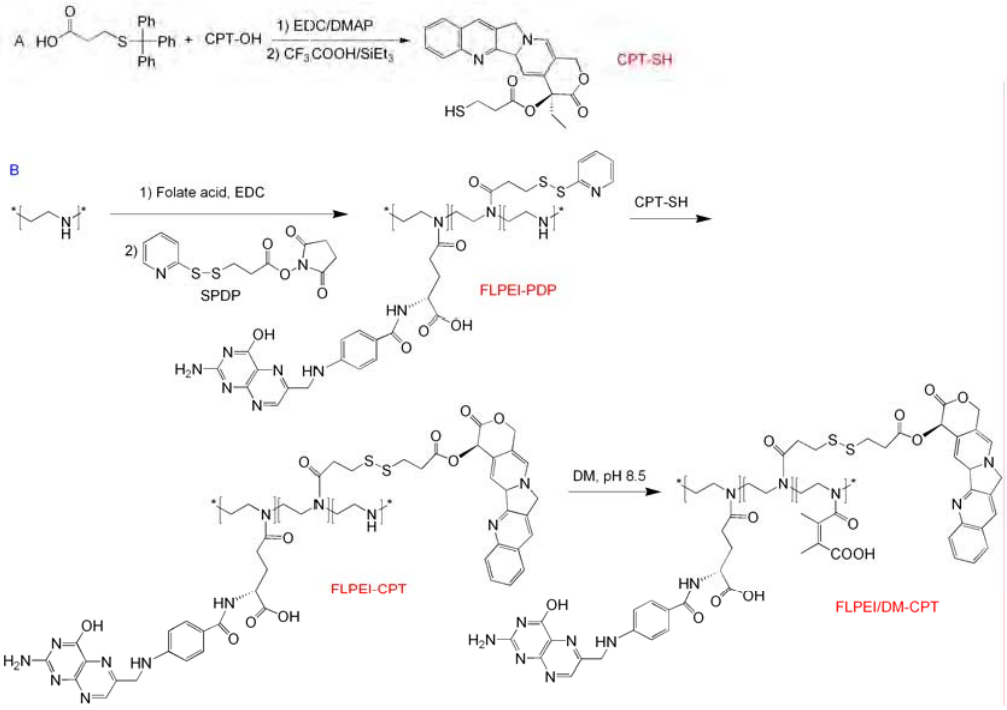
a. In vitro test drug release profile at pH 7.4 with or without GSH.
b. In vitro test cellular binding (competitive inhibition method)
c. In vitro test cellular uptake of TCRCs (flow cytometry, confocal laser-light scanning fluorescence microscopy).
d. In vitro test cytotoxicity to MCF7 breast cancer cells.
e. In vivo test biodistribution using mice.
f. In vivo test and compare anticancer activity using nude mice with sc tumors (systematic treatments).

Milestone 2: screening out the TCRC with the highest anticancer activity

Body

Synthesis and characterizations:

The synthesis of the FLPEI/amide-CPT is shown in Scheme 1. LPEI with molecular weight of 10,000 was synthesized according to the literature.[1] Folic acid is first introduced to LPEI by the reaction of its EDC-activated γ-carboxyl acid with the LPEI's secondary amine. Both UV and ^{1}H NMR were used to determine the average number of FA molecules attached to the LPEI.[2] Thiolated CPT-SH was then attached to the LPEI by a heterobifunctional coupling reagent SPDP. Subsequently, the LPEI amines were amidized by anhydrides in water at pH 8.5. The resulting amides were stable in a basic environment.



Scheme 1. The synthesis of CPT-SH (A) and FLPEI/DM-CPT (B).

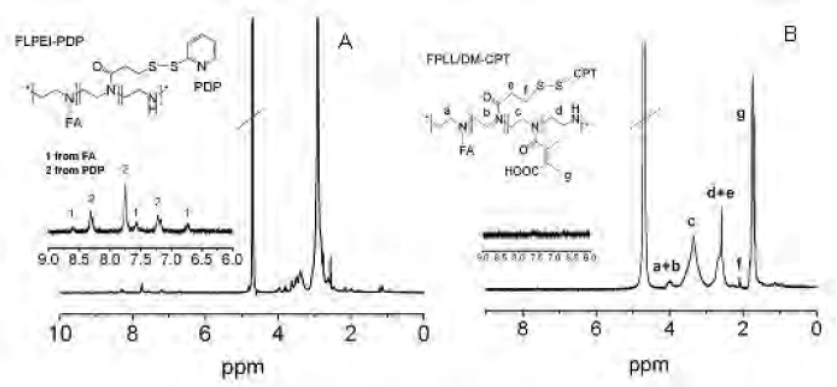


Figure 1. The ^{1}H NMR spectra of FLPEI-PDP (A) and FLPEI/DM-CPT (B).

The structures of the polymers were characterized by ^{1}H NMR (Figure 1). The CPT content was determined by HPLC.[3] The average number of attached FA molecules was calculated to be 1.5 per chain from the integration intensities of the methylene-proton in LPEI and the aromatic protons in the FA in the ^{1}H NMR spectrum, (Figure 1. A), which is in agreement with the result of the UV spectroscopy (1.8 folic acid groups per chain). The calculated amidization degree of the secondary amine was about 65% according to the integrals of the peak at 3.37 (CH_2CH_2NCO) and 2.62 ppm (CH_2CH_2NH) in the ^{1}H NMR spectrum (Figure 1. B). The peaks of the aromatic protons of CPT in the ^{1}H-NMR spectra were not clear probably due to the aggregation of the hydrophobic CPT moieties in water.

The content of CPT in FLPEI/DM-CPT was determined by HPLC after it was cleaved from the carrier in the presence of a 10-fold molar excess of dithiothreitol in a PBS buffer (pH 7.4) solution. The conjugate contained 5.0 wt% of CPT and less than 0.07 wt% free CPT.

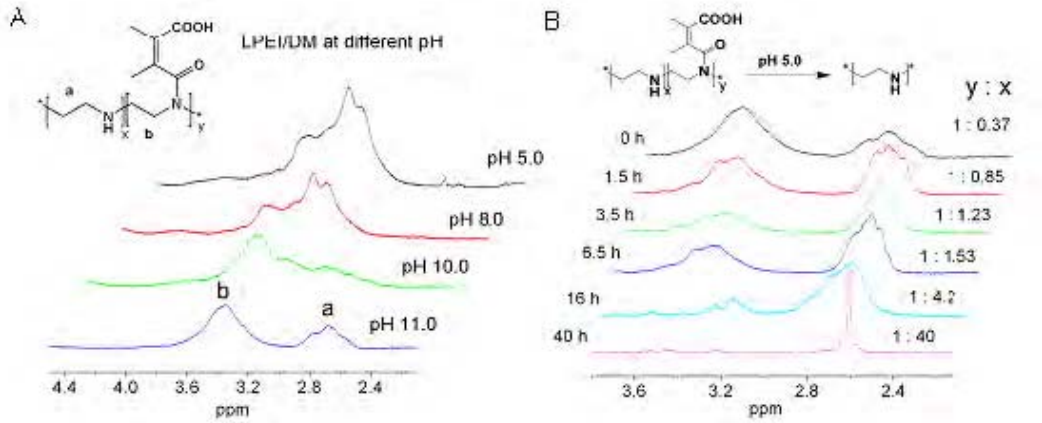


Figure 2. ^{1}H NMR spectra of LPEI/DM at different pH values (A) and the hydrolysis of LPEI/DM at pH 5.0 (B).

The pH-triggered hydrolysis of the β-carboxylic acid amide at pH of 7.4, 6.0 and 5.0 was monitored using ^{1}H NMR. The secondary amine in the LPEI would be partially charged at low pH and the chemical shifts of the methylene proton of CH_2CH_2NH and CH_2CH_2NCO overlapped. The peaks separated at acidic pH (e.g. pH 11.0) (Figure 2. A). Therefore, the hydrolysis of LPEI/DM was detected by ^{1}H NMR after the pH adjusted to 11.0. The chemical shifts of the methylene proton in CH_2CH_2NH changed from 3.4 to 2.6 ppm after the secondary amine was amidized. The typical intensity change of the two peaks during the hydrolysis of LPEI/DM at pH 5.0 is shown in Figure 2 B. With the hydrolysis progressed the intensity of the CH_2CH_2NCO peak decreased while that of the CH_2CH_2NH increased. The intensity ratio of the two peaks was used to calculate the percentage of the amide hydrolyzed. The hydrolysis rates of the LPEI/DM at different pH values are shown in Figure 3. The amide was acid-labile. The amides hydrolyzed quickly at an acidic pH (e.g., pH 5) but more slowly as the pH increased. The amides are stable in basic conditions (pH 11.0). It hydrolyzed 50% after approximately 6 h at pH 5.0 or 13 h at pH 6.0, and only 27% at pH 7.4 even after 40 h.

LPEI/DM has a β-carboxylic acid group and thus it should be negatively charged at pH 7.4. As the amides hydrolyzed to regenerate the secondary amines, the polymer should gradually become positively charged. The ζ-potentials of the LPEI/DM is shown in Figure 4. At pH 7.4, LPEI/DM had a ζ-potential of about -22 mV and remained negatively charged at pH 7.4 even after 40 h. It gradually became positive charged at pH 5.0 and 6.0. It took 1.2 h and 6.5 h for LPEI/DM to be positive charged at pH 5.0 and 6.0, respectively. These trends are consistent with the hydrolysis results determined by ^{1}H NMR in Figure 3. These data indicate that LPEI/DM was capable of negative-to-positive charge reversal.

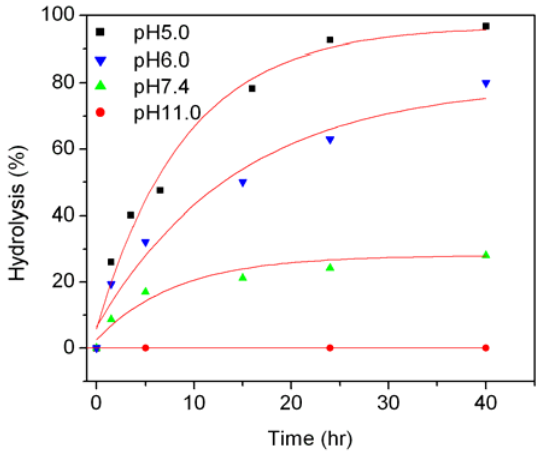


Figure 3. The hydrolysis of LPEI/DM at pH 5.0, 6.0, 7.4 and 11.0, respectively, detected by ^{1}H NMR.

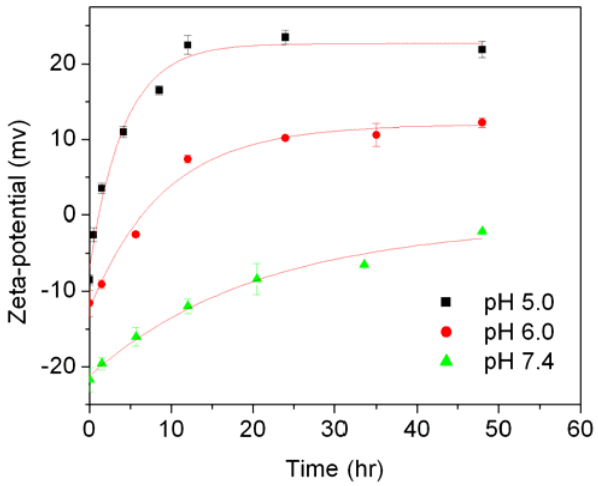


Figure 4. The time dependence of the Zeta-potential as a function of time for LPEI/DM (2 mg/ml) in 0.1 M PBS at pH 5.0 and 6.0, 7.4, respectively. (n=3)

In vitro characterizations

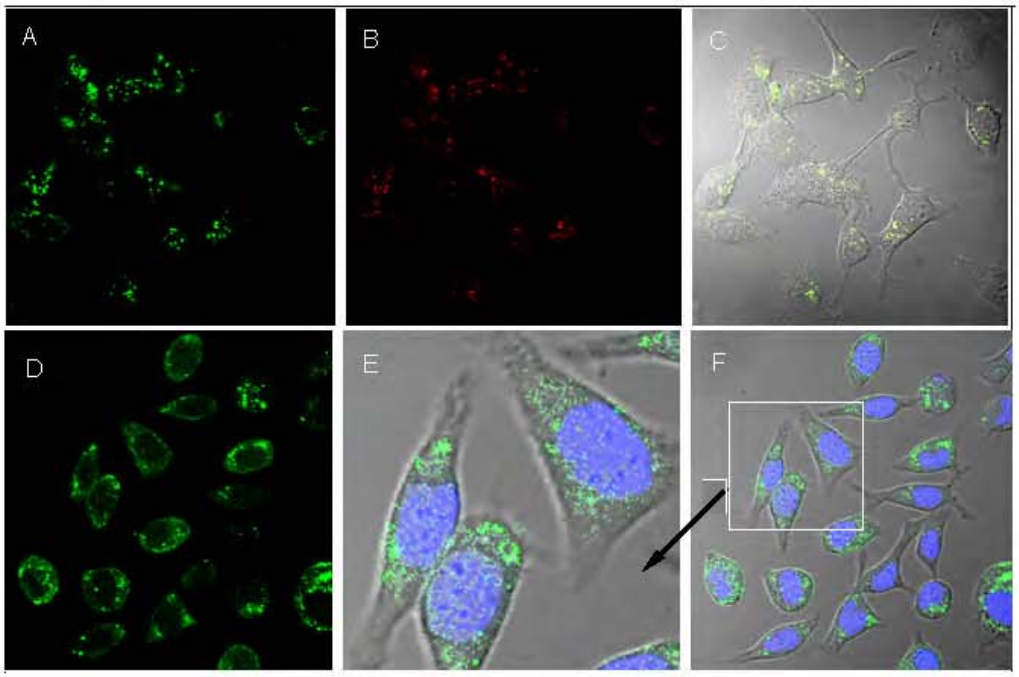


Figure 5. Localization in endosomes/lysosomes (upper panel) and nuclei (Lower panel) of FLPEI/DM-FITC in cancer cells observed by confocal florescence microscope. FPLPEI/DM-FITC (1.4 μM) was cultured with the cells for 24 h. Images of FLPEI/DM-FITC (A), LysoTracker(B), overlay of A and B with transmittance channel (C), FLPEI/DM-FITC (D), overlay of D with the images of the cells taken from the nuclear dye channel and transmittance channel (E), and the enlarged view (F).

For nuclear drug delivery, the conjugate must localize in acidic lysosomes for charge reversal, escape from the lysosomes, and subsequently traverse to the nucleus. The subcellular compartment labeling method was used to observe the subcellular distribution of FLPEI/DM-FITC using confocal microscopy (Figure 5. A-C). The fluorescence of FITC in FLPEI/DM-FITC was expressed as green. LysoTracker was used to label late endosomes/lysosomes and displayed as red. The overlay of the two fluorescence images shows yellow spots, indicating that FLPEI/DM-FITC indeed localized in late endosomes/lysosomes.

After being internalized, the conjugate is transferred to a late endosome/lysosome to regenerate its positive charges, and it is expected to lyse the lysosomal membrane to escape from it. The lysosomal-lysing ability of the carrier was estimated using hemolytic assay of red blood cells, a measure of a drug carrier's ability to rupture lysosomes.[4] RBCs were incubated with four different polymer concentrations in a range of 10–400 μg/ml (Table 1). Pristine LPEI caused 6.05% hemolysis at 10 μg/ml and 45.2% at higher concentrations in 2 h. LPEI/DM without pre-hydrolysis or pre-hydrolysis for 12 h at pH 6.0 showed no hemolytic ability at up to 400 μg/ml. LPEI/DM after pre-hydrolysis at pH 6.0 for 24 h showed a hemolytic activity similar to LPEI at

higher concentrations. The result demonstrates that LPEI/DM will have a similar lysosomal lysing ability to LPEI after regeneration in more acidic lysosomes (pH 4–5).

Table 1. Percentage release of hemoglobin (mean±SD) of sheep red blood cells after 120 min incubation with different concentrations of the polymer at 37°C (n=3)

Material	0.01mg/ml	0.05mg/ml	0.1mg/ml	0.4mg/ml
LPEI/DM	1.41±0.54	1.25±0.39	2.55±0.36	1.35±0.61
LPEI/DM (12 h at pH 6.0)	3.82±0.28	2.43±0.31	1.60±0.27	3.29±0.40
LPEI/DM (24 h at pH 6.0)	2.04±0.57	5.01±0.47	11.60±0.68	22.5±0.89
LPEI	6.05±0.98	9.83±0.41	24.7±0.32	45.2±0.97

The subsequent nuclear localization of LPEI/DM was confirmed by observing the colocalization of FITC-labeled FLPEI/DM (FLPEI/DM-FITC) and DRAQ-5 labeled nuclei (blue) using confocal microscopy (Figure 5. D–F). After a 24-h culture, some FLPEI-FITC (green) regenerated from FLPEI/DM-FITC in the lysosomes was clearly in the nuclei, suggesting that FLPEI/DM based conjugate was capable of accumulating in the nucleus.

The cytotoxicity of LPEI, LPEI/DM, free CPT and FLPEI/DM-CPT to MCF-7 breast cancer cells was evaluated using MTT assay. SKOV-3 ovarian cancer cells were also used to test its versatility. They were cultured with cells for 24 h, and then the cells were postcultured for 48 h to allow the damaged cells to undergo apoptosis. The results are presented in Figure 6. LPEI is very toxic due to its positive charges.[5] However, the negatively charged LPEI/DM had a very low cytotoxicity. There is still 75% cell viability even the concentration of LPEI/DM as high as 2 mg/ml. The IC_{50} of the CPT in the FLPEI/DM-CPT to SKOV-3 and MCF-7 cells is 3.2 μg/ml and 1.05 μg/ml, respectively. The FLPEI/DM-CPT showed obviously dose-dependent cytotoxicity. The enhanced cytotoxicity of CPT in the conjugate may be due to its nuclear drug delivery. CPT binds to and thereby stabilizes topoisomerase I, causing DNA damage and thus cell apoptosis.[6] Thus, CPT must traverse to the nucleus before it can elicit its drug action. The CPT conjugated to the carrier is brought to the nucleus by the LPEI regenerated in the lysosomes. The disulfide linker can be cleaved by a high concentration of glutathione (~0.5–10 mM) and thus CPT is released. The CPT released in the nucleus thus circumvents the drug-resistance mechanisms in the cell membrane and cytosol.

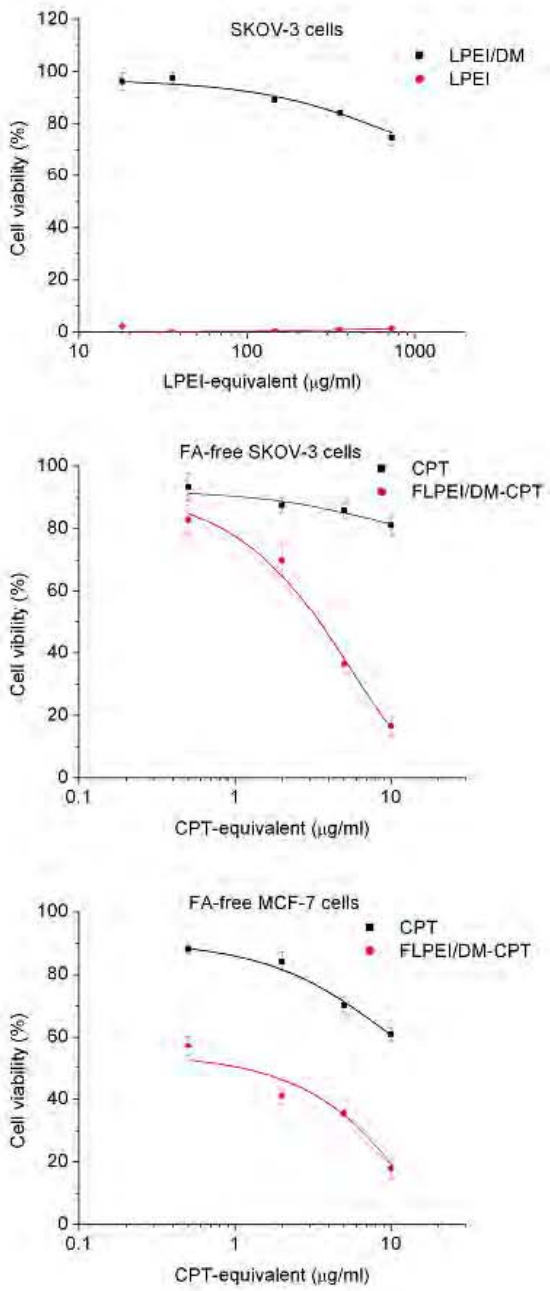


Figure 6. The cytotoxicity of LPEI, LPEI/DM, free CPT and FLPEI/DM-CPT respectively, estimated by MTT assay. Data represent mean ±SD, n=5.

Key Research Accomplishments

1) Synthesized the target polymer-drug conjugate and established the synthesis and characterization protocols
2) Proved that the nuclear drug delivery has a higher cytotoxicity to breast cancer cells.

Reportable Outcomes:

Era of Hope 2008:

TARGETED CHARGE-REVERSAL DRUG CARRIERS FOR NUCLEAR DRUG DELIVERY FOR BREAST CANCER CHEMOTHERAPY

Youqing Shen and William J. Murdoch

Abstract

Most cancer chemotherapy drugs target nuclear DNA to cause DNA damages and/or topoisomerase inhibition to induce cell death (apoptosis). In addition to the membrane-associated multidrug resistance, drug-resistant cancer cells also have many intracellular drug-resistance mechanisms to limit the access of drugs to the nucleus. Consequently, only a small percentage of drugs delivered into the cytosol finally reach the nucleus in drug-resistant cells. Thus, a drug carrier capable of localizing and releasing drugs directly into the nucleus would circumvent both the multidrug resistance and intracellular drug resistance mechanisms, leading to a high therapeutic efficacy.

Cationic polymers such as polyethyleneimine (PEI) and polylysine (PLL) can carry DNA across the cell membrane and harness the molecular motors to enter the nucleus. However, PEI and PLL have a rapid plasma clearance due to their positive charges from their amine groups.

An ideal regime would be to activate the cationic charges only in cancerous tissues or their intracellular compartments. Herein, we report drug carriers with a negative-to-positive charge reversal triggered by the solid tumor extracellular (pH <7) or lysosomal (4-5) acidity for nuclear drug delivery. The carriers are negatively charged in the bloodstream and have long circulation times. The drugs loaded in the charge-reversal carriers have a higher cytotoxicity than free drugs.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0645.

American Chemical Society National 2009 Spring Meeting

TARGETED CHARGE-REVERSAL LINEAR PEI FOR NUCLEAR DRUG DELVIER

Zhuxian Zhou, Edward A Van Kirk, William J Murdoch, Youqing Shen*

Abstract

We design and demonstrate the charge-reversal linear PEI drug conjugate for cancer cell nuclear delivery to enhance the drug's cytotoxicity. The designed charge-reversible linear PEI is capable of using in vivo since it has low interaction with the blood system and it will still disrupt

the lysosome membrane as soon as it regained to positive charged linear PEI. The disulfide bond linking the drug ensures the nuclear drug release. The MTT result shows an increased cytotoxicity of FLPEI/DM-CPT compared with the free drug.

Conclusion

We demonstrate a concept cancer cell-targeted charge-reversal drug conjugate for nuclear drug delivery to enhance the drug's cytotoxicity. LPEI's positive charges are masked by converting them to latent amides, significantly inhibiting its ability to interact with cells. Once the amidized LPEI is transferred to the cell lysosomes, the amides hydrolyze to regenerate the amines and thus the LPEI's nuclear-localization ability is recovered. By functionalizing the amidized LPEI with folic acid targeting moieties and an anticancer drug via an intracellular-cleavable disulfide bond, the drug can be efficiently shipped to the cell nucleus and ensuring high cytotoxicity.

Reference

1. Brissault, B.; Kichler, A.; Guis, C.; Leborgne, C.; Danos, O.; Cheradame, H., Synthesis of linear polyethylenimine derivatives for DNA transfection. *Bioconjugate. Chem.* **2003**, 14, 581.
2. Majoros, I.J.; Thomas, T.P.; Mehta, C.B.; Baker, J.r.J.R. Poly(amidoamine) dendrimer-based multifunctional engineered nanodevice for cancer therapy. *J. Med. Chem.* **2005**, 48, 5892.
3. Henne, W.A.; Doorneweerd, D.D.; Hilgenbrink, A.R.; Kularatne, S.A.; Low, P.S. Synthesis and activity of a folate peptide camptothecin prodrug. Bioorg. *Med. Chem. Lett.* **2006**, 16, 5350.
4. Murthy, N.; Robichaud, J.R.; Tirrell, D.A.; Stayton, P.S.; Hoffman, A.S. The design and synthesis of polymers for eukaryotic membrane disruption. *J. Control. Rel.* 1**999**, 61, 137.
5. Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* **2003**, 24, 1121.
6. Wall, M.E.; Wani, M.C. Camptothecin and taxol: discovery to clinic. *Cancer. Res.* **1995**, 55, 753.